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**INTRODUCTION.** We are examining apoptotic pathways that may be altered in human mammary epithelial cells as a consequence of the overexpression of prostaglandin G/H synthase 2. We have shown that human mammary epithelial cells express high levels of prostaglandin G/H synthase, are resistant to ionizing radiation-induced apoptosis, but do apoptose when exposed to the broad spectrum kinase inhibitor staurosporine or to DNA damage caused by hydrogen peroxide or mitomycin C. We have found that PGHS activity does not affect the apoptotic response of human mammary epithelial cells, nor does PGHS activity affect the cell cycle response after DNA damage. We have also demonstrated that mitomycin C, but not ionizing radiation, can induce the p53 protein in human mammary epithelial cells.

**BODY.** An understanding of early stages of cancer development is essential due to the correlation between patient survival and early detection, and investigation of the initial stages of cancer may suggest novel markers for patient screening or targets for new therapeutics. The majority of research on apoptosis has been carried out in hematopoietic or fibroblast cell lines, however, it is becoming increasingly evident that there may be important distinctions in how breast epithelial cells modulate their response to DNA damage or other insults. Since the majority of human cancers are derived from epithelial cells, the characterization of apoptosis, which may remove cells with intrinsic genomic instability, in this specific cell type should provide important information on the etiology of human breast cancer. Establishment of an epithelial tissue culture system was an important step toward characterizing this cell type. Breast epithelium normally undergoes cyclic apoptosis during the menstrual cycle, and a potential deregulation of this normal physiological occurrence could lead to the development of neoplasia. Immortalization itself may be an early event in the development of cancer, providing a pool of cells with altered proliferative capacity. We have available a normal human mammary epithelial cell line as well as an immortalized, non-tumorigenic, cell line derived from the normal cell line through treatment with the chemical carcinogen benzo(a)pyrene. The use of benzo(a)pyrene-immortalized mammary cells is an important distinction in these studies: these cells present a more realistic representation of developing breast neoplasms than virally-immortalized cell lines.

Cancer cells can be distinguished based on their lack of differentiation, inappropriate proliferation, and immortal phenotype. The development of a cancer is a multi-step, lengthy process and can be separated into three steps; initiation, promotion, and progression. The first step, initiation, occurs when a DNA damaging agent causes a mutation that is fixed into the genome as the cell replicates. Promotion is a complex process, but it clearly involves a clonal expansion of initiated cells that have developed a selective growth advantage. The last step, progression, is a neoplastic evolution driven by genomic instability and resulting in eventual metastasis of the cancer cells. However, normal cells possess mechanisms to prevent the development of cancer. One important component in this defense is the ability of cells to control their progression through the cell cycle. A G1 checkpoint allows cells to repair DNA damage before it becomes incorporated into the genome during S phase as a mutation. Alternatively, cells that have extensive damage or have bypassed the normal cellular checkpoints may be removed via the apoptotic pathway. Apoptosis is often referred to as "programmed cell death" and involves selective removal of cells. Characteristic morphological changes seen in apoptosis are a loss of cell volume, condensed chromatin, and plasma membrane blebbing. The nucleus breaks up into membrane-enclosed fragments, followed by the cell itself. The membrane-bound fragments are rapidly phagocytosed by the surrounding cells without triggering an inflammatory response.

Apoptosis is also an important mechanism in normal developmental processes such as embryogenesis, the immune response, and hormone-dependent structural changes in the breast and prostate. In the embryo, apoptosis acts to remove selected subpopulations of

cells in various organs. This process removes autoimmune T lymphocytes during thymocyte maturation in the thymus and a large number of neurons in the developing brain (reviewed in [1]). In the mature organism, cytotoxic T cells are thought to kill some virally infected cells via apoptosis [2, 3], and the depletion of CD4<sup>+</sup> T cells during HIV infection may be due to uncontrolled apoptosis [4, 5]. Following castration, the rat prostate undergoes involution and this process requires the programmed cell death of androgen-deprived cells [6]. Apoptosis is a normal event in the lactating mammary gland [7] and also serves to remodel breast tissue following weaning [8, 9]. Moreover, in normal breast tissue apoptosis shows a cyclic trend, peaking near the end of the menstrual cycle [10].

Proteins which mediate the apoptotic response in the breast include p53, bcl-2 and bax. p53 is a transcription factor that has been shown to inhibit cell proliferation and suppress transformation and tumorigenesis. Perturbation of p53 signaling pathways may give affected cells a growth advantage and contribute to neoplasia: accordingly p53 is mutated or lost in over 50% of human cancers. It has also been suggested that loss of wild-type p53 could contribute to the formation of radio- and chemotherapy-resistant tumors, however this paradigm has recently been called into question [11]. Nelson and Kastan [12] found that DNA strand breaks are sufficient for increasing p53 protein levels, and Kastan *et al.* [13] have demonstrated that fibroblasts from p53-null mice lack a G1 checkpoint induced by ionizing radiation. It has been suggested that p53 may also be involved in a G2 delay [14], although this may not be a direct effect [15]. One negative regulator of p53 is the proto-oncogene MDM2, which was identified by its ability to enhance tumorigenic potential when overexpressed [16]. It was demonstrated by Momand *et al.* [17] that p53-mediated transcriptional activation can be inhibited by mdm2 oncogene product binding to the p53 protein. Mdm2 mRNA levels can be induced by wild-type p53 [18] and this may form a negative feedback loop, whereby excessive p53 levels lead to an increase in mdm2 protein production and subsequent inactivation of the p53 protein [19]. As would be expected, DNA damage can cause induction of Mdm2 in a p53-dependent fashion [20, 21]. Another apparent modulator of p53 signaling is the ataxia telangiectasia (AT) gene product. AT is an autosomal recessive disease that is marked by hypersensitivity to ionizing radiation, radioresistant DNA synthesis, and a progressive cerebellar ataxia with degeneration of Purkinje cells. There is also an increase in cancer risk associated with AT, and women who are heterozygous for AT have up to a five-fold greater risk for breast cancer [22]. The ionizing radiation induced increase in p53 levels is delayed in cells from AT patients [13]. In addition, other components of the p53-dependent DNA damage response are also altered in AT cells: Waf1, Mdm2 and Gadd45 all exhibit delayed or decreased induction in AT cells exposed to ionizing radiation [23]. A gene mutated in all four complementation groups of AT has been identified, and a partial cDNA clone isolated [24].

Bcl-2 was identified several years ago as a gene overexpressed in human follicular lymphoma due to a translocation event that places it under the influence of regulators of the IgH gene [25, 26]. In normal human breast tissue, bcl-2 protein levels vary throughout the menstrual cycle and are highest at the end of the luteal phase [27]. It is also commonly overexpressed in breast cancer [28]. Bcl-2 can also protect cells exposed to apoptosis-inducing stimuli, not all of which are p53-dependent [29]. In cultured human mammary epithelial cells, bcl-2 overexpression inhibits cell death due to confluence or serum starvation [30], while overexpression of Bax accelerates apoptotic death. The activity of bcl-2 is thought to be mediated through a protein-protein interaction with bax, which is a member of the same protein family. Bax forms homodimers or heterodimers with bcl-2 *in vivo*, and it has been suggested that the bcl-2/bax ratio may act as a determinant of apoptosis [31]. Supporting this theory is the finding that human breast cancer cell lines that are resistant to apoptosis contain a low level of bax- $\alpha$  (a splicing variant of bax) when compared to non-malignant epithelial cell lines [32].

Another potential mediator of programmed cell death in human breast cells is p21<sup>Waf1/Cip1</sup>. p21<sup>Waf1/Cip1</sup> is a 21 kDa protein that can be induced in a p53 dependent manner in G1 arrest through inhibition of cyclin-dependent kinases [33] and apoptosis [34]. Overexpression of p21<sup>Waf1/Cip1</sup> in human breast cancer cells results in morphological changes and induces growth arrest and apoptosis [35]. p21<sup>Waf1/Cip1</sup> expression can be induced by a variety of agents including UV [36], oxidative stress [37], and kinase inhibitors [38]. However, not all of these responses are p53 dependent [37, 39, 40].

It has been shown that an enzyme involved in the inflammation process may also have an effect on cell cycle and apoptosis. Prostaglandin G synthase (also referred to as cyclooxygenase) and prostaglandin H synthase are two enzymatic reactions catalyzed by the same protein, a homodimer of approximately 70 kDa subunits (hereafter referred to as PGHS). PGHS catalyzes the conversion of arachidonic acid to the hydroperoxide PGG<sub>2</sub>, then the reduction of PGG<sub>2</sub> to the alcohol PGH<sub>2</sub>. Inhibitors of PGHS can decrease the risk of colon cancer [41], and can also reduce the number and size of adenomatous polyps [42]. Elevated levels of prostaglandins are found in both lung and colon cancers [43, 44], and the mitogen-inducible form of prostaglandin G/H synthase (PGHS2) is expressed at high levels in a large number of colon cancers [45, 46]. Overexpression of PGHS2 increases resistance to apoptotic stimuli in rat intestinal epithelial cells with a concurrent increase in bcl-2 levels [47], and alterations in PGHS2 expression may also affect the cell cycle [48]. A relationship between p53 expression and PGHS2 expression has recently been described [49]. The PGHS enzymes may play a more indirect role in carcinogenesis: they can also metabolize the procarcinogen benzo[a]pyrene to the active carcinogen benzo[a]pyrene-7,8-diol-epoxide [50-53].

The metabolic activation of benzo[a]pyrene (B[a]P) by human mammary epithelial cells (HMEC) produces not only B[a]P-DNA adducts, but also oxidative DNA damage [54]. In normal HMEC184 cells, the production of this oxidative damage is transient and returns to undetectable levels when B[a]P treatment is ended. We have found that the induction of the oxidative DNA damage occurs via the arachadonic acid cascade. Treatment of cells with B[a]P in the presence of inhibitors of PGHS, indomethacin or ibuprofen, reduces the levels of one type of oxidative base modification, thymine glycols (Table 1). A human mammary epithelial cell line immortalized by multiple treatments with B[a]P, HMEC184B5, maintains elevated levels of thymine glycols even in the absence of B[a]P treatment. The basal levels of thymine glycols in these cells is equivalent to the cell receiving between 30-40 Gy of ionizing radiation. The production of the reactive oxygen species in these cells is also dependent on the arachadonic acid cascade since treatment with either indomethacin or ibuprofen significantly reduces the amount of thymine glycols (Table 1). The high background of oxidative DNA damage in the immortalized HMEC184B5 cells was not due to slower removal of this base damage relative to the normal HMEC184 cells. When thymine glycols are induced by treatment with hydrogen peroxide, both HMECs removed thymine glycols with a similar efficiency (data not shown). We investigated whether the high levels of thymine glycols in the immortalized breast cells could be due to the induction of the pathway which induces this oxidative DNA damage when the cells are treated with B[a]P, namely the arachadonic acid cascade. We examined the expression of the inducible form of PGHS, PGHS2, in the immortalized HMEC184B5 cells. Subbaramaiah et. al. [55] have described enhanced transcription of PGHS2 in transformed mammary epithelial cells, and we now report that normal and immortalized human mammary epithelial cells constitutively express high levels of PGHS2 (Figure 1A).

Since high levels of PGHS2 can protect rat intestinal epithelial cells from apoptosis [47], one prediction would be that the HMEC would be resistant to apoptosis-inducing agents. We therefore exposed HMEC184B5 cells to ionizing radiation to induce apoptosis. Apoptosis was measured by visualizing the production of a laddering of DNA fragments

180 base pairs or multiple in size, which is characteristic of apoptosis. Following treatment with 8 or 16Gy of ionizing radiation, no induction of DNA ladders was detectable by either 24 or 46 hours post-treatment (Figure 2B). However, when HMEC184B5 are exposed to increasing doses of staurosporine, a protein kinase inhibitor shown to induce apoptosis, DNA fragmentation is detected (Figure 2C). Our results with ionizing radiation suggest that that HMEC184B5 cells cannot undergo apoptosis via a p53-dependent pathway. However, DNA damage-induced apoptosis is not necessarily always mediated through p53 [29]. By comparison, staurosporine appears to act through a p53-independent pathway [56]. Staurosporine, a non-specific serine/threonine kinase inhibitor, can disrupt the cell cycle and is thought to cause apoptosis by interfering with the timely activation of cell cycle components [57]. Treatment of cells with staurosporine can lead to induction of p21<sup>Waf1/Cip1</sup> [38].

### **HYPOTHESIS/PURPOSE**

Our preliminary results show that immortalized HMEC184B5 cells express high levels of PGHS2. In addition, these cells maintain extremely elevated levels of oxidative DNA damage, a situation one would expect to lead to apoptosis, yet appear to be unable to undergo apoptosis when exposed to ionizing radiation. **We hypothesize that the overexpression of PGHS2 prevents immortalized mammary epithelial cells from undergoing apoptosis via a p53-dependent pathway.** If our hypothesis is correct, then we expect that immortalized cells will not undergo apoptosis when exposed to agents which induce apoptosis via a p53-dependent pathway. Conversely, these cells should be able to apoptose when exposed to agents which induce apoptosis via a p53-independent pathway. We also expect the p53-independent induction of apoptosis to occur at a different stage in the cell cycle than does the p53-dependent pathway. Finally, we expect that either eliminating or reducing PGHS2 activity in the immortalized cells should restore their ability to undergo apoptosis.

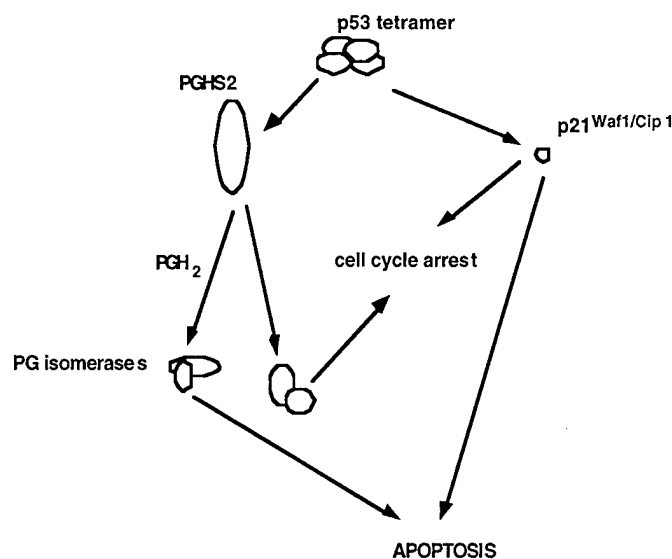
**Results.** We have shown that HMEC constitutively express PGHS2 (Figure 1) and do not respond to IR by undergoing apoptosis (Figures 2A, 2B), although they are capable of inducing apoptosis when exposed to staurosporine (Figure 2C). This information, along with the data that show PGHS isozymes produce oxidative DNA damage in HMEC (Table 1), led us to hypothesize that PGHS2 expression would protect HMEC from DNA damaging agents that induce apoptosis through a p53-dependent pathway. However, treatment of HMEC with an inhibitor of PGHS isozymes (indomethacin) does not activate an apoptotic pathway after IR (Figure 3), nor does it affect the G1 and G2/M cell cycle arrest following IR (Figures 4, 5). Although these cells exhibit a prolonged arrest in G1 and G2/M, p53 is not induced (Figure 6). Hydrogen peroxide does activate an apoptotic pathway in HMEC (Figure 7), but this apoptotic response and the corresponding cell cycle arrest are not affected by inhibition of PGHS isozymes (Figures 8, 9). The DNA crosslinking agent mitomycin C also causes apoptosis in HMEC (Figure 10) and, like the apoptosis induced by hydrogen peroxide, this response occurs regardless of PGHS activity (Figure 11). Accordingly, a cytotoxicity assay shows that there is no difference in cell survival following mitomycin C treatment in cells treated with indomethacin (Figure 12). Interestingly, this apoptotic response was associated with an increase in the levels of p53 (Figure 13).

We have attempted to develop human mammary epithelial cells that contain an antisense-expressing construct to attenuate the high levels of endogenous prostaglandin G/H synthase 2. This was to provide a second means for modulating PGHS2 levels in the HMEC, and thus control for possible non-specific actions caused by the NSAID indomethacin. HMEC 184B5 cells were transfected with either a control vector (pCB7) or a vector containing the cDNA sequence of prostaglandin G/H synthase 2 in the antisense orientation, driven by a CMV promoter and a hygromycin B selection cassette (pCB7COX2AS). Several hygromycin-resistant clones for each transfection were isolated,



and were expanded for freezedown and analysis. The clones isolated show no aberrant morphology or growth characteristics when compared to wildtype HMEC 184B5 cells. Unfortunately, when these clones were analyzed by Western analysis, probing for PGHS2 expression, we found that there was no significant change in PGHS2 expression (Figure 14).

**Discussion.** The data we have obtained suggest that there is no role for PGHS isozymes in modulating the DNA-damage apoptotic response in human mammary epithelial cells. PGHS2 has been described as a nuclear matrix-associated protein [58, 59], and certain prostaglandin products can cause G1 arrest in cells [60-62], although this response is mediated through  $p21^{Waf1/Cip1}$  [63, 64], it may be p53 independent [65]. The role of PGHS2 in this arrest is suggested by the report of DuBois *et. al.* [48] where a G1 delay was observed in cells overexpressing PGHS2. The details of how prostaglandin products induce  $p21^{Waf1/Cip1}$  are still unclear, but prostaglandin receptors have been described in the nucleus [66-68], [69]. A possible scenario is that the induction of  $p21^{Waf1/Cip1}$  or the apoptotic response depends on the differential production of certain prostaglandin products such as  $PGA_2$  or  $PDJ_2$ , while at the same time down-regulating other eicosanoid products. This model would involve p53 signaling through parallel pathways involving  $p21^{Waf1/Cip1}$  and PGHS2 and its downstream isomerases (See Diagram below). Thus the decision to undergo programmed cell death may not be directly attributable to PGHS2, but to the prostaglandins that are generated by isomerases from  $PGH_2$  supplied by PGHS2. While there are DNA-damage response pathways that may involve p53 and PGHS2, or both, these pathways may feed through  $p21^{Waf1/Cip1}$ . Thus the question remains: why doesn't PGHS activity affect cell cycle or apoptotic responses in HMEC? A possible explanation may be found in that these cells maintain high levels of p53 and PGHS2 even in the absence of any DNA damage. Therefore, since p53 and its downstream target  $p21^{Waf1/Cip1}$  are not induced by ionizing radiation in HMEC, apoptosis cannot occur regardless of PGHS2 status. In the case of hydrogen peroxide or mitomycin C, there is an induction of p53, however the already high levels of p53 and p21 in the HMEC preclude any PGHS2 involvement by allowing the cell to bypass it and go through a p53/ $p21^{Waf1/Cip1}$  pathway. Alternatively, a more simple explanation is that this cell type may not be capable of producing the isomerases that convert  $PGH_2$  from PGHS2 into pro-apoptotic prostaglandins.



It would appear that in HMEC, PGHS inhibition has no effect on other DNA damage responses such as the cell cycle arrest. HMEC undergo a G1 and a G2/M arrest in

response to ionizing radiation or hydrogen peroxide, regardless of PGHS activity. Our data seem to suggest that HMEC undergoing apoptosis do so at the G1/S phase border, but this is not certain. The difference in cell death seen between ionizing radiation and hydrogen peroxide are perplexing, since hydrogen peroxide produces damage similar to that caused by ionizing radiation, yet the cells undergo apoptosis following peroxide treatment but not radiation. Ionizing radiation, but not hydrogen peroxide, produces double strand breaks, and our results suggest that double strand breaks can interfere with the apoptotic program following DNA damage in this cell type. We have observed an induction of p53 following hydrogen peroxide treatment (data not shown) as well as after mitomycin C exposure. One possibility is that the induction of p53 and activation of an apoptotic pathway is secondary to damage recognition by a DNA repair complex. The presence of double strand breaks caused by ionizing radiation creates a lesion where this repair complex is forced to dissociate with the DNA double helix, and thus the signal for apoptosis cannot be transduced. Since double strand breaks are not produced by hydrogen peroxide, and are produced only indirectly after mitomycin C treatment, the apoptotic signal can be sent and results in the accumulation of p53.

Our original hypothesis that PGHS2 prevented immortalized HMEC from undergoing apoptosis has now been shown not to be the case. We have observed that both the normal HMEC 184 and immortalized HMEC 184B5 cells respond in a similar manner to DNA damage, and must therefore conclude that, while the human mammary epithelial cells do possess an intact DNA damage apoptotic pathway, PGHS2 does not play a role in modulating this response. It may still be possible for PGHS2 to be involved in apoptotic pathway in certain other cell types *in vivo* or *in vitro*, and this may depend on the p53 or p21<sup>Waf1/Cip1</sup> status of the cells. We will now focus our research into determining the role of p53 and/or p21<sup>Waf1/Cip1</sup> in the apoptotic response of human mammary epithelial cells exposed to DNA damage.

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### **KEY RESEARCH ACCOMPLISHMENTS**

We have drawn the following conclusions from this project:

- Human mammary epithelial cells maintain an intact apoptotic pathway that can be induced with the protein kinase inhibitor staurosporine or by DNA damage produced by hydrogen peroxide or mitomycin C, but not by ionizing radiation;
- This DNA-damage induced apoptosis can be induced in a time- and dose- dependent manner in both normal and immortalized HMEC;
- PGHS2 activity does not affect the HMEC apoptotic or cell cycle response to DNA damage;
- HMEC do induce p53 after certain types of DNA damage.

**REPORTABLE OUTCOMES**

The following reportable outcomes are a direct result of the funding of this project:

- Two abstracts presented at national meetings;
- One manuscript under preparation;
- One Ph.D. degree expected in December 1999;
- A postdoctoral research position applied for based on the training supported by this award



**Expression of PGHS2 and alterations in apoptotic pathways: cell cycle consequences.** Robert W. Fisher and Steven A. Leadon, Department of Radiation Oncology and the Curriculum in Toxicology, University of North Carolina, Chapel Hill, NC, 27514-7512 USA.

One objective of our studies is to provide a better understanding of how breast epithelial cells maintain their genomic integrity in response to DNA damage. Cells can respond to genomic damage by either delaying transit through the cell cycle or by undergoing programmed cell death. Consequently, the loss or attenuation of cell cycle checkpoints or apoptotic pathways may result in an increased number of cells with DNA damage retained in the general cell population, thus increasing the probability of engendering genomic instability. Metabolic activation of the carcinogen benzo[a]pyrene generates reactive oxygen species that damage DNA, and in normal human mammary epithelial cells (HMEC184), the production of reactive oxygen species is transient and returns to basal levels when benzo[a]pyrene treatment is ended. However, a human mammary epithelial cell line (HMEC184B5) immortalized by multiple treatments with benzo[a]pyrene maintains a high oxidative background even in the absence of benzo[a]pyrene. This oxidative damage appears to be produced by the arachidonic acid cascade. We found that normal HMEC184 and immortalized HMEC184B5 cells express high levels of an arachidonic acid metabolizing enzyme, the mitogen-inducible form of prostaglandin G/H synthase (PGHS2). It has been shown that overexpression of PGHS2 alters the cell cycle response and increases resistance to apoptotic stimuli in rat intestinal epithelial cells. **We hypothesize that activation of the arachidonic acid cascade in the HMEC results in the production of reactive oxygen intermediates and a block in the p53-dependent apoptotic response.** We find that both HMEC184 and HMEC184B5 cells do not undergo apoptosis when exposed to ionizing radiation, but do when exposed to staurosporine. These results indicate that the p53-dependent apoptotic response is blocked in the HMEC, while a p53-independent apoptotic pathway remains intact in these cells. We will further examine whether HMEC exposed to apoptotic stimuli undergo a cell cycle delay and determine the stage in the cell cycle that precedes the apoptotic response induced by staurosporine. The results of these experiments should provide important new information on early events in the transformation of normal breast cells as well as potential targets for chemotherapeutic intervention in breast cancer.

(Supported by U.S. Army Medical Research Fellowship 17-97-1-7156).

**Prostaglandin synthase isozymes do not modulate DNA-damage induced apoptosis in human mammary epithelial cells.** Robert W. Fisher and Steven A. Leaden. Curriculum in Toxicology and the Department of Radiation Oncology. University of North Carolina, Chapel Hill, NC USA.

An objective of our laboratory is to provide a better understanding of how breast epithelial cells maintain their genomic integrity in response to DNA damage. Cells can respond to genomic damage by either delaying transit through the cell cycle or by undergoing apoptosis. Consequently, the loss or attenuation of cell cycle checkpoints or of apoptotic pathways may result in an increased number of cells with DNA damage retained in the general cell population, thus increasing the probability of engendering genomic instability. One enzyme that may play a role in modulating cell cycle arrest and apoptosis is prostaglandin G/H synthase. It has been shown that cells that overexpress the inducible prostaglandin G/H synthase 2 have a delayed transit through G1 and are resistant to apoptosis. Additionally, nonspecific inhibition of prostaglandin G/H synthase isozymes by nonsteroidal antiinflammatory drugs can lead to apoptosis in some cell types. We have previously shown that human mammary epithelial cells express constitutively high levels of prostaglandin G/H synthase 2 and do not respond to ionizing radiation by undergoing apoptosis. Inhibition of prostaglandin synthase activity through the nonsteroidal anti-inflammatory agent indomethacin did not affect the HMEC's response to ionizing radiation. We have shown that HMEC can respond to DNA damage by undergoing apoptosis, and that this response is not modulated by nonspecific inhibition of PGHS isozymes. We conclude that PGHS2 is not an active component of the apoptotic response in this human mammary epithelial cell culture system.

(Supported by Department of Defense Breast Cancer Research Program Fellowship 17-97-1-7156)

**Table 1. Inhibition of oxidative DNA damage in HMEC by inhibitors of PGHS.**

Cell Culture	Treatment	Thymine Glycols <sup>a</sup>
184 (Normal)	None	ND <sup>b</sup>
	B[a]P (2.5 ug/mL)	12.3
	B[a]P+indomethacin (25 uM)	2.0
	B[a]P+ibuprofen(25 uM)	0.9
184B5 (immortal)	None	8.2
	indomethacin (25 uM)	0.5
	ibuprofen(25 uM)	0.4

<sup>a</sup> per 10<sup>6</sup> base pairs

<sup>b</sup> Not Detectable

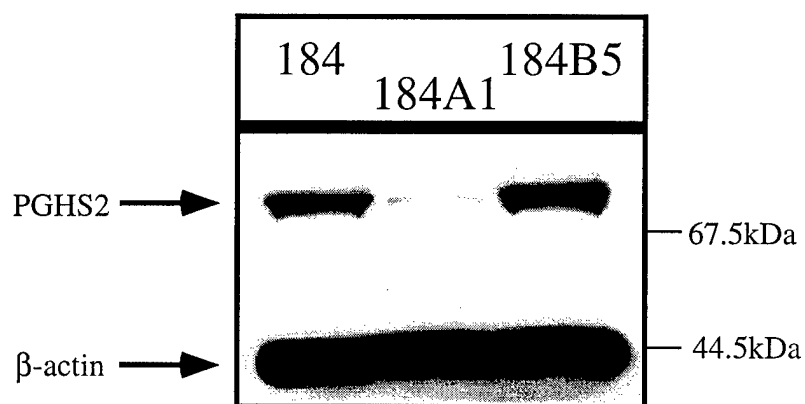


Figure 1. PGHS2 is constitutively expressed in HMEC. Protein was isolated from exponentially growing asynchronous HMEC and 10  $\mu$ g analyzed for PGHS2 expression.  $\beta$ -actin is shown as a loading control.

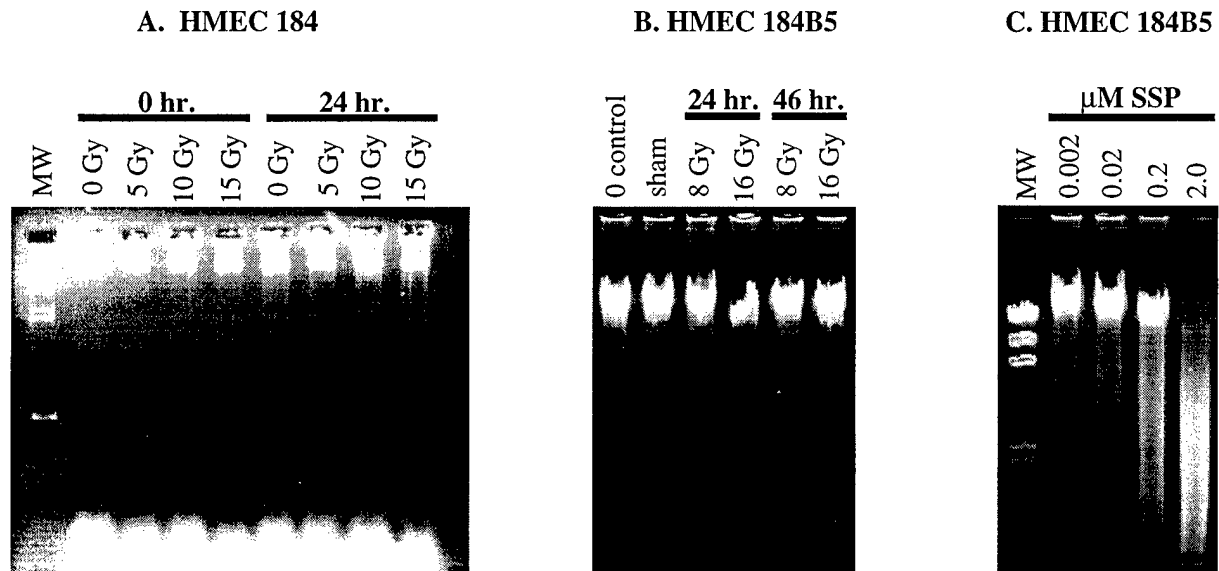


Figure 2. Staurosporine, but not ionizing radiation, induces apoptosis in human mammary epithelial cells. HMEC 184 cells exposed to various doses of ionizing radiation were collected 24 hours later; A. HMEC 184B5 cells were sham irradiated or exposed to 8 or 16 Gy of ionizing radiation. Cells were collected at 24 and 46 hours post irradiation; C. HMEC 184B5 cells were exposed to various doses of staurosporine for 24 hours, then collected; C. For all panels DNA was extracted from attached and detached cells and analyzed on a 2% agarose gel

indomethacin

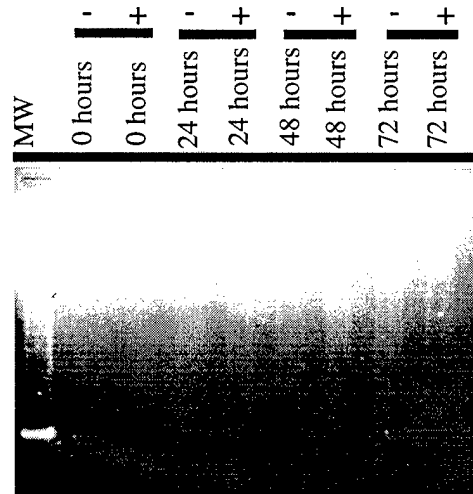
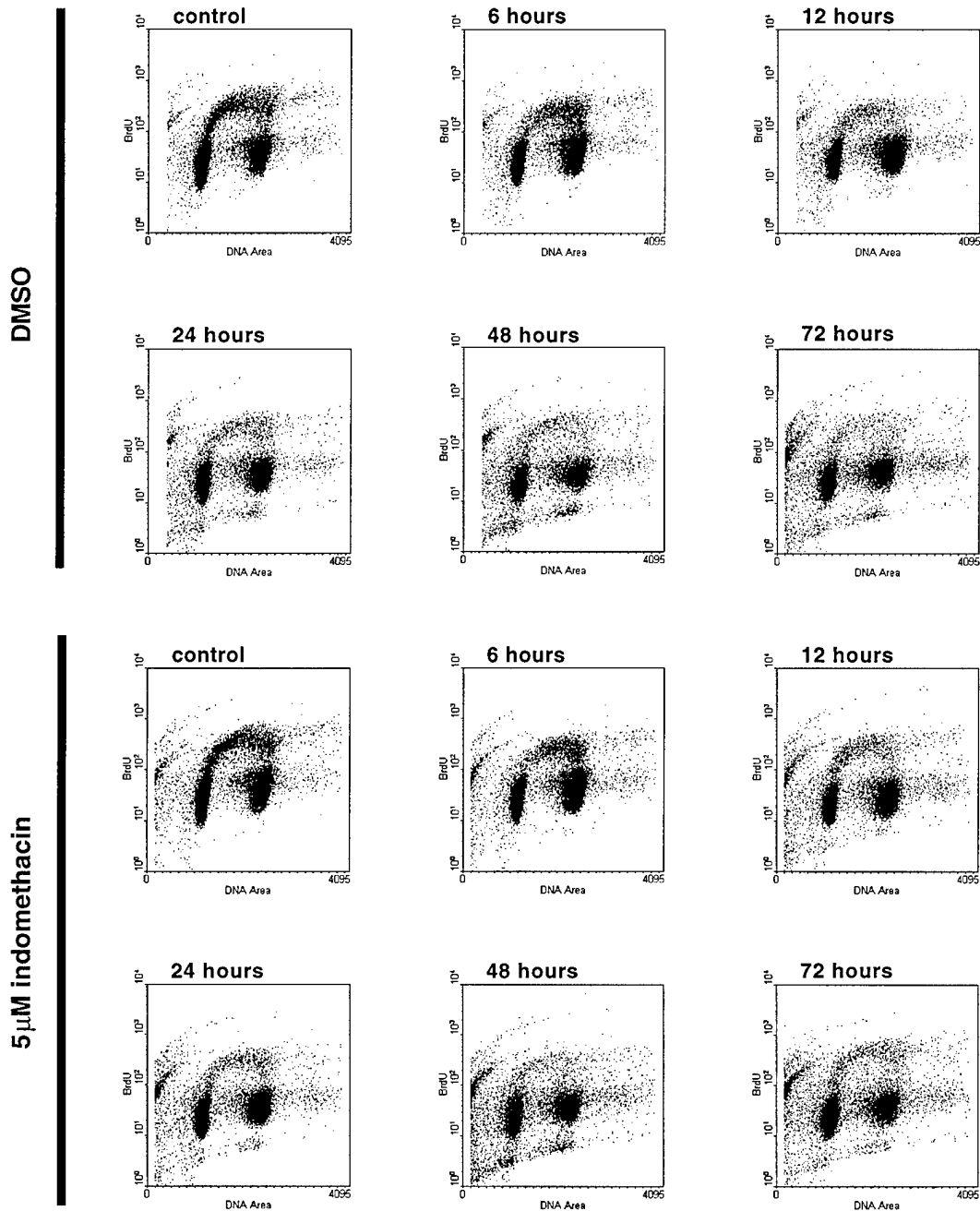


Figure 3. PGHS inhibition does not sensitize HMEC 184 cells to ionizing radiation-induced apoptosis. HMEC 184 cells were pretreated with DMSO (0.01%) or indomethacin (5 $\mu$ M) for 4 hours, then exposed to 10 Gy of ionizing radiation. At the indicated times DNA was extracted from attached and detached cells and analyzed on a 2% agarose gel



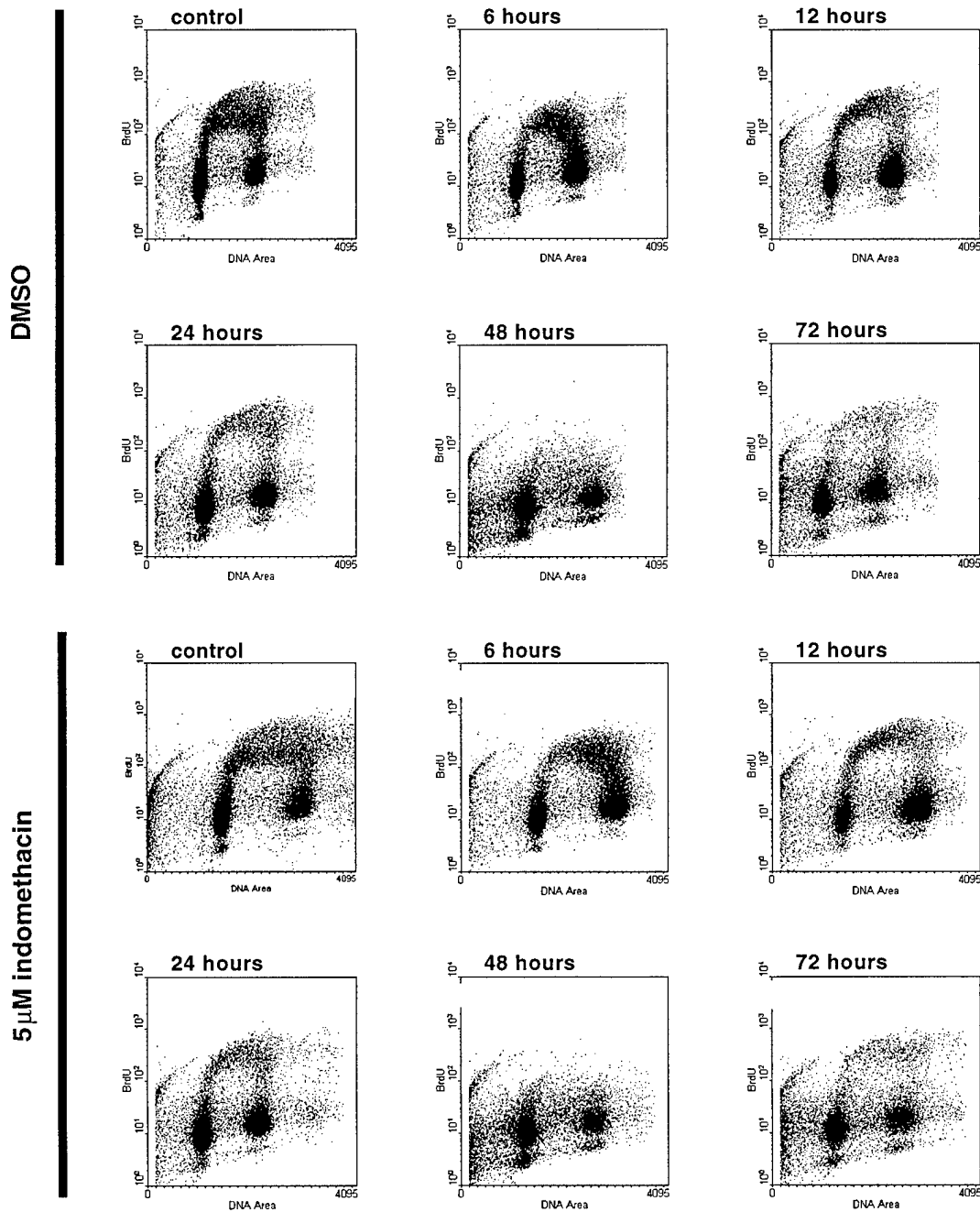


Figure 5. PGHS inhibition does not affect the cell cycle response of HMEC 184B5 cells following exposure to ionizing radiation. HMEC 184B5 cells were pretreated for 4 hours with DMSO (0.1%) or indomethacin (5 $\mu$ M), then exposed to 10 Gy of ionizing radiation. Cells were collected at the times shown and analyzed by dual-parameter flow cytometry as described in the Materials and Methods.

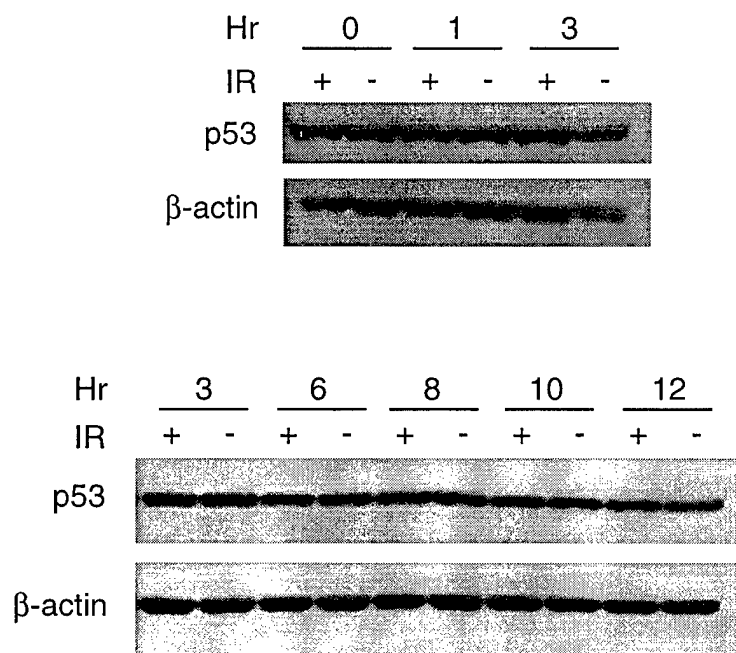
**HMEC 184**

Figure 6. p53 is not induced by ionizing radiation in HMEC 184 cells. HMEC 184 were sham-irradiated or exposed to 4 Gy of ionizing radiation, then collected for western analysis at the indicated times.  $\beta$ -actin is shown as a loading control. From Meyer et. al., *Oncogene*, in press (used with permission).



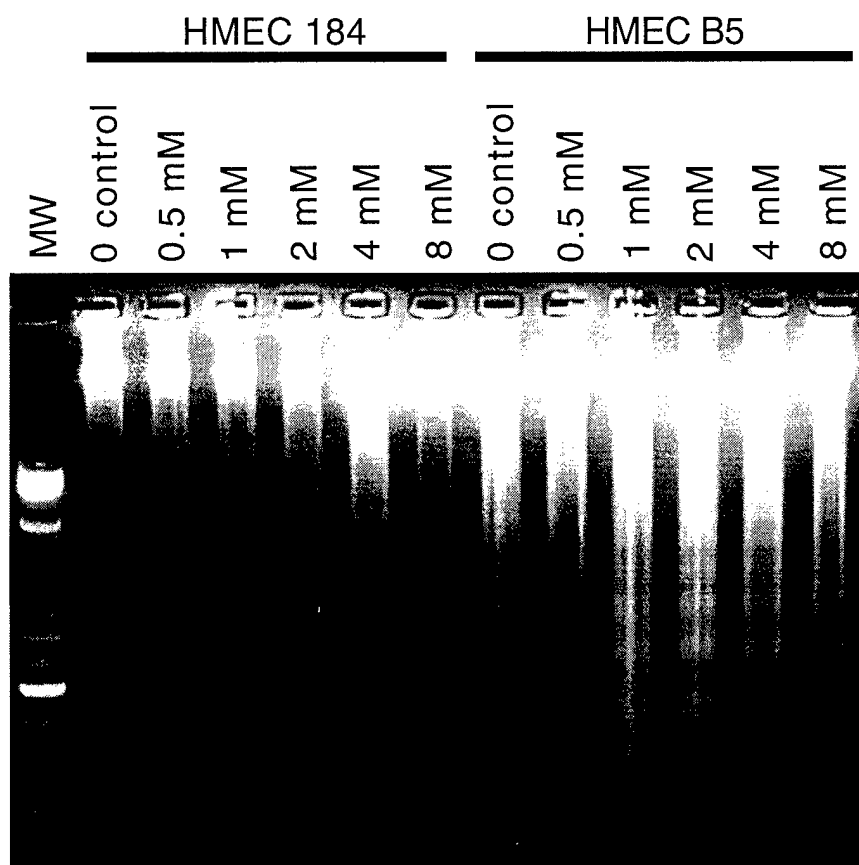


Figure 7. Hydrogen peroxide causes apoptosis in HMEC. HMEC 184 or 184B5 cells were exposed to the indicated dose of hydrogen peroxide for 24 hours. DNA was isolated from attached and detached cells and analyzed on a 2% agarose gel.

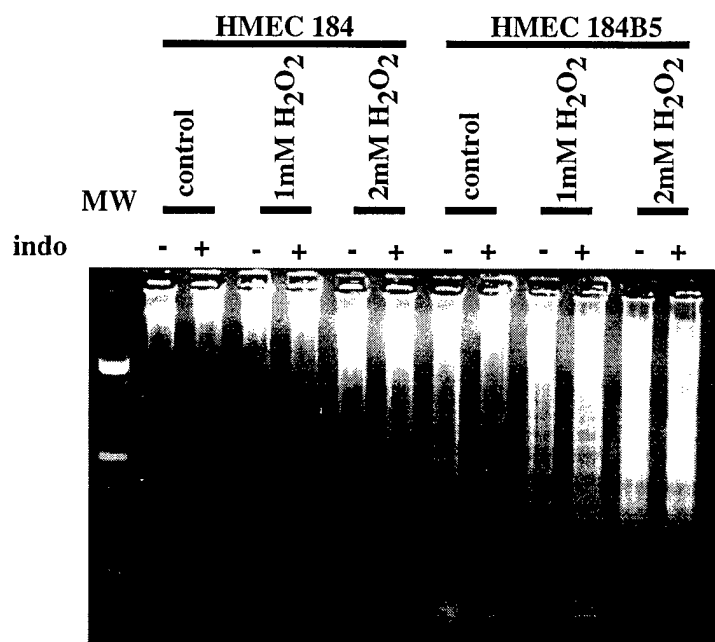


Figure 8. PGHS inhibition does not affect hydrogen-peroxide- induced apoptosis in human mammary epithelial cells. HMEC 184 or HMEC 184B5 were pretreated with DMSO (0.1%) or indomethacin (25 $\mu$ M) for 4 hours, then exposed to the indicated dose of hydrogen peroxide for 24 hours. Cells were collected and DNA isolated from attached and detached cells, then analyzed on a 2% agarose gel.

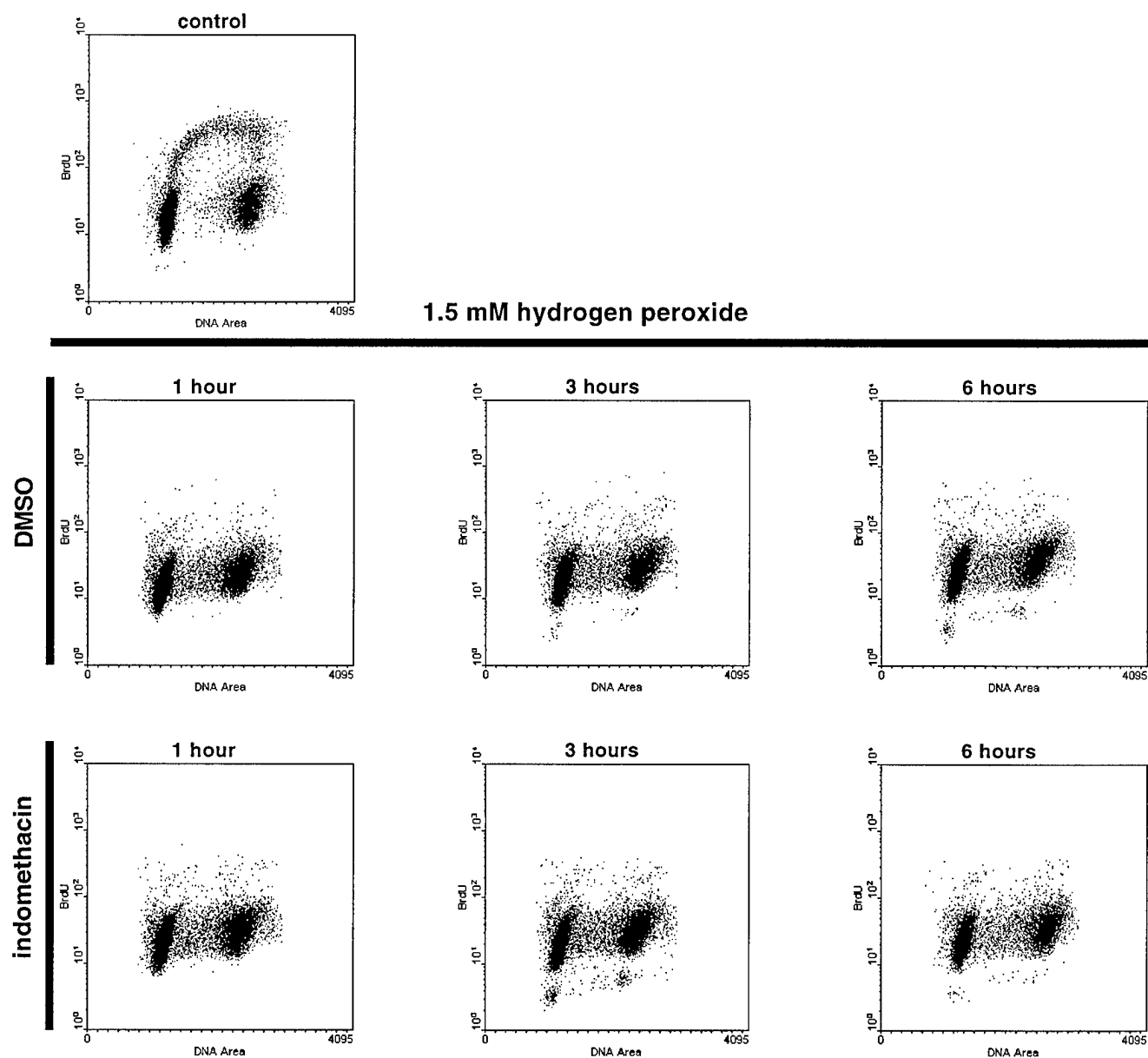


Figure 9. PGHS inhibition does not affect the cell cycle response of HMEC 184 cells following exposure to hydrogen peroxide. HMEC 184 cells were pretreated for 4 hours with DMSO (0.1%) or indomethacin (25 $\mu$ M), then exposed to 1.5mM hydrogen peroxide. Cells were collected at the times shown and analyzed by dual-parameter flow cytometry as described in the Materials and Methods.

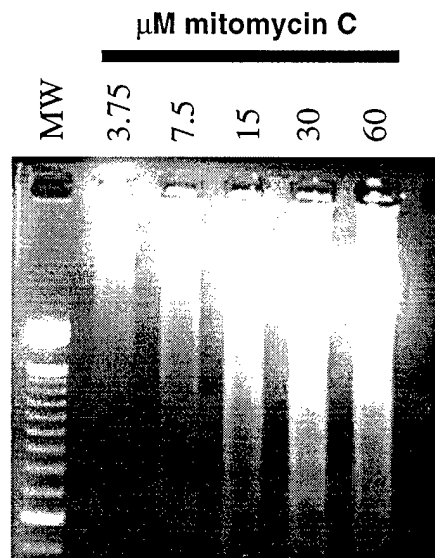


Figure 10A. Mitomycin C causes apoptosis in HMEC. HMEC 184 cells were exposed to the indicated dose of mitomycin C for 24 hours. DNA from attached and detached cells was collected and analyzed on a 2% agarose gel.

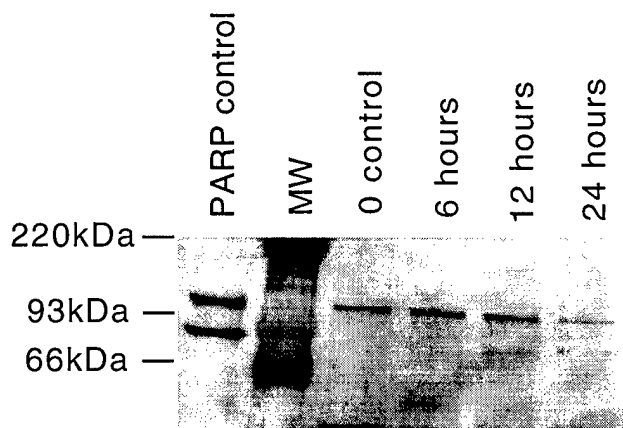


Figure 10B. Mitomycin C causes PARP cleavage in HMEC. HMEC 184 cells were exposed to 15  $\mu\text{M}$  mitomycin C for 0 to 24 hours. Protein from attached and detached cells was electrophoresed on a 4-20% SDS-PAGE gel, transferred to nitrocellulose, and probed with an anti-PARP antibody.

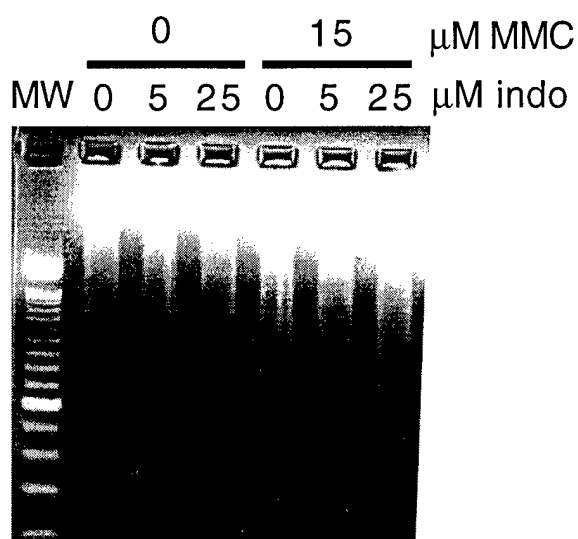


Figure 11. PGHS inhibition does not sensitize HMEC 184 cells to mitomycin C-induced apoptosis. HMEC 184 cells were exposed to 0 or 15  $\mu\text{M}$  mitomycin C for 24 hours in the presence of 0 or 25  $\mu\text{M}$  indomethacin. DNA was then extracted from attached and detached cells and analyzed on a 2% agarose gel.

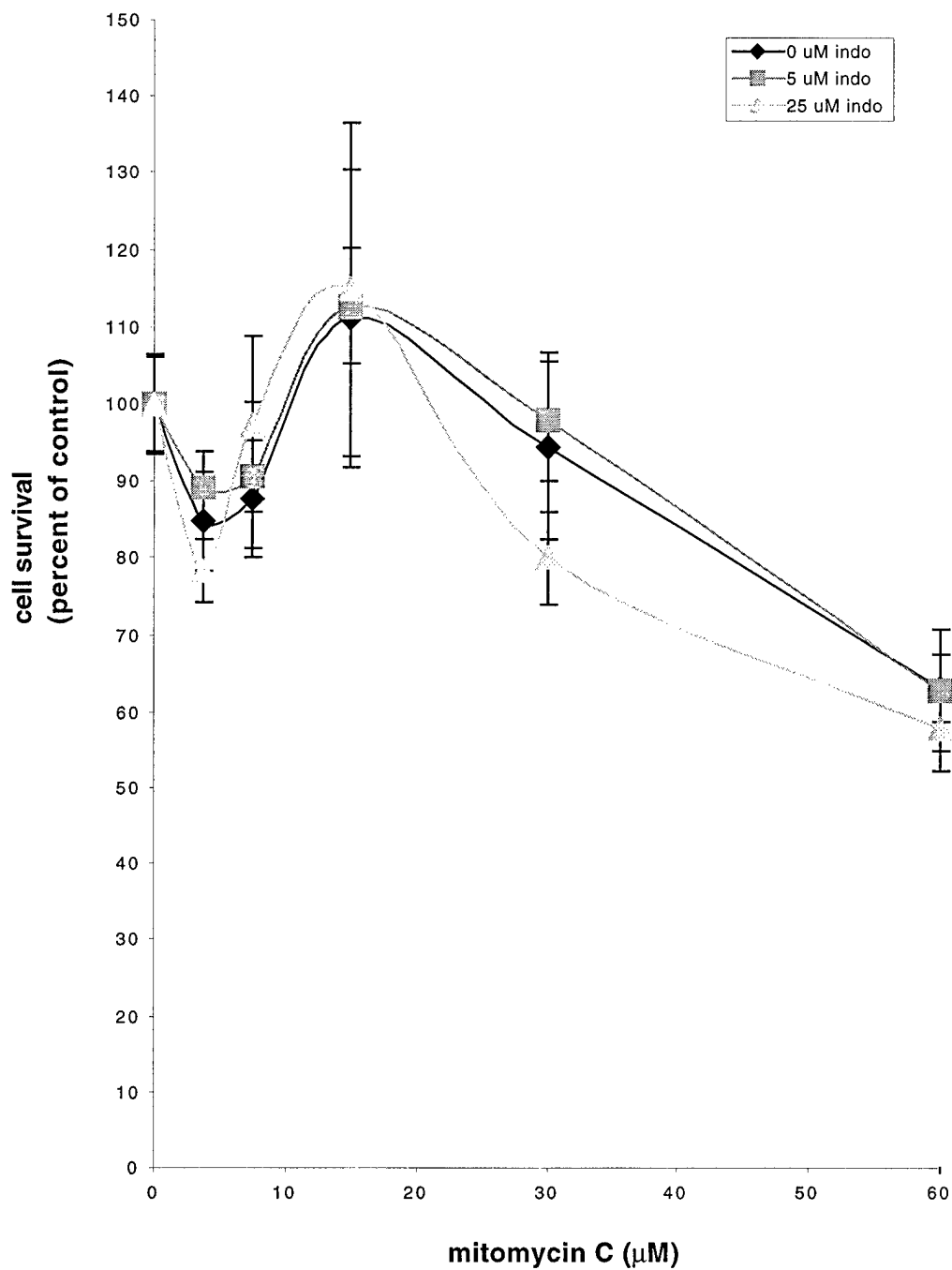


Figure 12. PGHS inhibition has no effect on cell survival after exposure to mitomycin C. HMEC 184 cells were pretreated with DMSO (0.1%) or increasing doses of indomethacin for 4 hours, then exposed to various doses of mitomycin C for 24 hours. Cell survival was assayed using the Cell Titer 96AQ protocol (Promega).

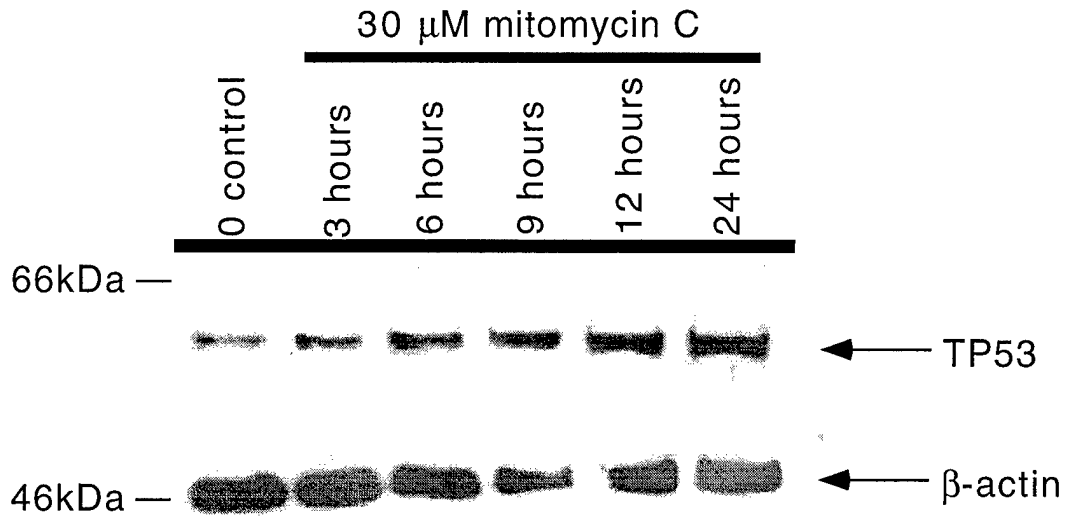


Figure 13. Mitomycin C exposure induces p53 in HMEC 184 cells. HMEC 184 cells were exposed to 30  $\mu$ M mitomycin C for the indicated times. Protein from attached and detached cells was electrophoresed on a 10%SDS-PAGE gel, transferred to nitrocellulose, and probed with an anti-TP53 antibody.

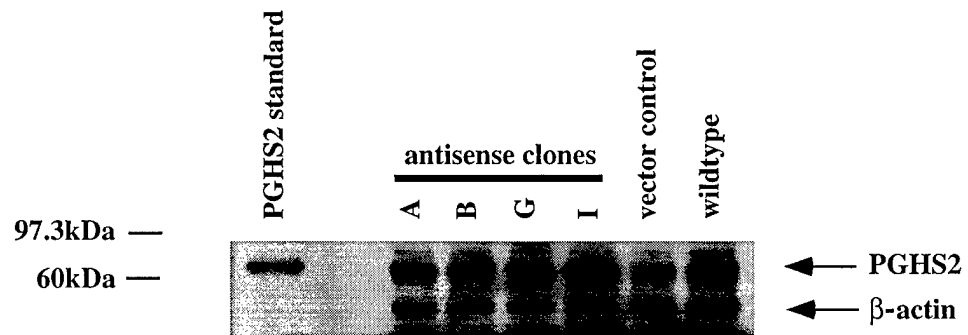


Figure 14. PGHS2 expression in HMEC 184B5 cells transfected with vector alone, or an antisense-expressing construct. Protein was isolated from exponentially growing hygromycin-resistant HMEC 184B5 clones and 20  $\mu$ g analyzed for PGHS2 expression.  $\beta$ -actin is shown as a loading control.



**ABBREVIATIONS:**

DNA: deoxyribonucleic acid  
HMEC: human mammary epithelial cell  
MMC: mitomycin C  
PGHS: prostaglandin G/H synthase  
SSP: staurosporine